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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/19, C07K 14/52, C12Q 1/68,</b> <b>C07K 16/24</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/33990</b> <b>(43) International Publication Date:</b> 8 July 1999 (08.07.99)
<b>(21) International Application Number:</b> PCT/US98/26546 <b>(22) International Filing Date:</b> 14 December 1998 (14.12.98)  <b>(30) Priority Data:</b> 60/068,955 30 December 1997 (30.12.97) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 60/068,955 (CIP) Filed on 30 December 1997 (30.12.97)  <b>(71) Applicant (for all designated States except US):</b> CHIRON CORPORATION [US/US]; 4560 Horton Street – R440, Emeryville, CA 94608 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> CHEN, Tseng-hui, Timothy [US/US]; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US). POT, David [US/US]; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US). KASSAM, Altaf [US/US]; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US).	<b>(74) Agents:</b> POTTER, Jane, E., R. et al.; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US).  <b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> HUMAN CXC CHEMOKINE ( <i>Tim-1</i> )  <b>(57) Abstract</b>  A human CXC chemokine gene termed <i>Tim-1</i> and its expression products can be used to design therapeutic tools for treating inflammation due to stimuli such as heart attacks and stroke, infection, physical trauma, UV or ionizing radiation, burns, frostbite, or corrosive chemicals. Regulation of immune responses against tumors, angiogenesis, and cellular migration, can also be effected using <i>Tim-1</i> therapeutic tools.		

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## HUMAN CXC CHEMOKINE (*Tim-1*)

### TECHNICAL AREA OF THE INVENTION

The invention relates to the area of chemokines. More particularly, the invention  
5 relates to chemokine regulation of inflammation, immune responses against tumors, angiogenesis, and chemotaxis.

### BACKGROUND OF THE INVENTION

Chemokines play an important role in the regulation of inflammation, immune  
10 responses against tumors, angiogenesis, and chemotaxis. Manipulation of chemokine gene expression can be used, for example, to regulate these processes in mammalian cells and to prevent or treat inflammation. Thus, there is a need in the art for the identification of mammalian chemokine genes.

### SUMMARY OF THE INVENTION

15 It is an object of the invention to provide reagents and methods for regulating inflammation, immune responses against tumors, angiogenesis, and chemotaxis. These and other objects of the invention are provided by one or more of the embodiments described below.

20 One embodiment of the invention is an isolated human Tim-1 protein having an amino acid sequence which is at least 85% identical to SEQ ID NO:2. Percent identity between the first and second human Tim-1 proteins is determined using a Smith-

Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

Another embodiment of the invention is an isolated polypeptide comprising at least 8 contiguous amino acids as shown in SEQ ID NO:2.

5 Even another embodiment of the invention is a Tim-1 fusion protein comprising a first protein segment and a second protein segment fused together by means of a peptide bond. The first protein segment consists of at least 8 contiguous amino acids of a human Tim-1 protein having an amino acid sequence as shown in SEQ ID NO:2.

10 Yet another embodiment of the invention is a preparation of antibodies which specifically bind to a human Tim-1 protein having an amino acid sequence as shown in SEQ ID NO:2.

Still another embodiment of the invention is a cDNA molecule which encodes a human Tim-1 protein having an amino acid sequence which is at least 85% identical to SEQ ID NO:2. Percent identity is determined using a Smith-Waterman homology search  
15 algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

Even another embodiment of the invention is a cDNA molecule which encodes at least 8 contiguous amino acids of SEQ ID NO:2.

20 Another embodiment of the invention is a cDNA molecule which comprises at least 12 contiguous nucleotides of SEQ ID NO:1.

Yet another embodiment of the invention is a cDNA molecule which is at least 85% identical to the nucleotide sequence shown in SEQ ID NO:1. Percent identity is determined using a Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

25 Still another embodiment of the invention is an isolated and purified subgenomic polynucleotide comprising a nucleotide sequence which hybridizes to SEQ ID NO:1 after washing with 0.2X SSC at 65 °C. The nucleotide sequence encodes a Tim-1 protein having the amino acid sequence of SEQ ID NO:2.

Even another embodiment of the invention is a construct comprising a promoter and a polynucleotide segment encoding at least 8 contiguous amino acids of a human Tim-1 protein as shown in SEQ ID NO:2. The polynucleotide segment is located downstream from the promoter. Transcription of the polynucleotide segment initiates at the promoter.

5        Another embodiment of the invention is a host cell comprising a construct. The construct comprises a promoter and a polynucleotide segment which encodes at least 8 contiguous amino acids of a human Tim-1 protein having an amino acid sequence as shown in SEQ ID NO:2.

10       Yet another embodiment of the invention is a recombinant host cell comprising a new transcription initiation unit. The new transcription initiation unit comprises in 5' to 3' order: an exogenous regulatory sequence, an exogenous exon, and a splice donor site. The new transcription initiation unit is located upstream of a coding sequence of a *Tim-1* gene having a coding sequence as shown in SEQ ID NO:1. The exogenous regulatory sequence controls transcription of the coding sequence of the *Tim-1* gene.

15       Still another embodiment of the invention is a polynucleotide probe comprising at least 12 contiguous nucleotides of SEQ ID NO:1.

20       The present invention thus provides the art with a novel human CXC chemokine gene called *Tim-1* chemokine (*Tim-1*). The *Tim-1* chemokine gene and protein can be used, *inter alia*, to design therapeutic tools for treating or preventing inflammation, regulating immune responses against tumors, regulating angiogenesis, and affecting chemotaxis.

#### **DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

25       A novel human CXC chemokine gene (*Tim-1*) has been identified. The *Tim-1* chemokine gene, protein, and mRNA can be used, *inter alia*, to design therapeutic tools for treating or preventing inflammation, regulating immune responses against tumors, regulating angiogenesis, and affecting chemotaxis.

Human *Tim-1* mRNA is expressed at high levels in brain, prostate, thymus, and peripheral blood lymphocytes. Placenta, liver, and kidney express low amounts of *Tim-1*

mRNA. *Tim-1* is involved in a variety of biological activities, including the chemoattraction of cells (chemotaxis), induction of inflammatory responses, regulation of immune responses against tumors, and angiogenic activity.

Full-length human Tim-1 chemokine protein (Tim-1) has the amino acid sequence disclosed in SEQ ID NO:2. Any naturally occurring biologically active variants of this sequence which occur in human tissues are within the scope of this invention. Naturally occurring biologically active variants of Tim-1 have similar biological activities to a Tim-1 protein having the sequence shown in SEQ ID NO:2, such as inflammation-inducing activity, chemotaxis of cells such as neutrophils, lymphocytes, hemopoietic progenitors, monocytes, or natural killer cells, regulation of immune responses against tumors, and angiogenic activity.

Tim-1 polypeptides (fragments) differ in length from full-length Tim-1 protein and comprise 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 45, 50, 75, 80, 90, or 100 or more contiguous amino acids of a Tim-1 protein. One class of fragments of particular interest are those that retain heparin binding. Such binding can be determined according to the assay in Luster *et al.*, J. Exp. Med. 182: 219 (1995). The heparin binding portion of Tim-1 is located at the  $\alpha$ -helical portion, specifically, the C-terminus of the Tim-1. Heparin binding fragments can include those with amino acids 85 to 111 of Tim-1. Preferably, the fragments comprise amino acids 88 to 103 of Tim-1; even more preferably, amino acids 89 to amino acids 102 of Tim-1.

Another class of Tim-1 fragments are those that comprise a three stranded  $\beta$ -sheet of the Tim-1. Such fragments can be useful as inhibitors to prevent dimerization of Tim-1. Inhibition of dimerization of Tim-1 to modulate dimerization of a Tim-1 receptor can reduce signal transduction and biological activity. Fragments exhibiting a  $\beta$ -sheet can include amino acids 37 to 84 of Tim-1; preferably, such fragments include amino acid 39 to 80 of Tim-1; even more preferably, such fragments comprise amino acids 42 to 72 of Tim-1. Assays to test for dimerization ability of fragments are described in Burrows *et al.*, *Biochem* 33: 12741 (1994) and Zhang *et al.*, *Mol. Cell. Biol.* 15: 4851 (1995).

Other fragments of interest are those comprising the N-terminal loop of the Tim-1. Such fragments can include, for example, amino acids 13-41, 26-39, or 29-37 of Tim-1.

Variants of Tim-1 protein and Tim-1 polypeptides can also occur. Tim-1 variants can be naturally or non-naturally occurring. Naturally occurring Tim-1 variants are found  
5 in humans or other species and comprise amino acid sequences which are substantially identical to the amino acid sequence shown in SEQ ID NO:2. Non-naturally occurring Tim-1 variants which retain substantially the same biological activities as naturally occurring Tim-1 variants are also included here.

Preferably, naturally or non-naturally occurring Tim-1 variants have amino acid  
10 sequences which are at least 85%, 90%, or 95% identical to amino acid sequences shown in SEQ ID NO:2 and have similar biological properties, including inflammation-inducing activity and the ability to effect chemotaxis of cells such as neutrophils, lymphocytes, hemopoietic progenitors, monocytes, or natural killer cells. More preferably, the molecules are at least 98% or 99% identical. Percent identity between a Tim-1 protein  
15 variant and SEQ ID NO:2 is determined using a Smith-Waterman algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1. The Smith-Waterman homology search algorithm is taught in Smith and Waterman, *Adv. Appl. Math.* (1981) 2:482-489.

Preferably, amino acid changes in Tim-1 variants are conservative amino acid  
20 changes, *i.e.*, substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and  
25 uncharged polar (glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids.

It is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar

replacement of an amino acid with a structurally related amino acid will not have a major effect on the biological properties of the resulting Tim-1 variant. Properties and functions of Tim-1 variants are of the same type as a Tim-1 protein or polypeptide comprising amino acid sequences of SEQ ID NO:2, although the properties and functions of variants can  
5 differ in degree. Whether an amino acid change results in a functional Tim-1 variant can readily be determined by assaying activities of the Tim-1 chemokine variant, as described below.

Tim-1 activity includes chemotactic, immunological, biological, receptor binding, and signal transduction abilities. Tim-1 affects chemotaxis of neutrophils, lymphocytes,  
10 tumor-infiltrating lymphocytes, hemopoietic progenitors, monocytes, and natural killer cells. The ability of Tim-1 variants to affect chemotaxis of neutrophils can be assayed as described in Walz *et al.* (1987), *Biochem. Biophys. Res. Commun.* 149: 755; Yoshimura *et al.* (1987), *Proc. Natl. Acad. Sci. USA* 84: 9233, and Schroder *et al.* (1987), *J. Immunol.* 139: 3474. Chemotaxis of lymphocytes can be assayed as described in Larsen *et al.*,  
15 *Science* 243: 1464: (1989) and Carr *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 3652 (1994).

Assays for chemotaxis of tumor-infiltrating lymphocytes are described in Liao *et al.* (1995), *J. Exp. Med.* 182: 1301; for hemopoietic progenitors, in Aiuti *et al.* (1997), *J. Exp. Med.* 185: 111; for monocytes, in Valente *et al.* (1988), *Biochem.* 27: 4162; and for natural killer cells, in Loetscher *et al.* (1996), *J. Immunol.* 156: 322, and in Allavena *et al.*  
20 (1994), *Eur. J. Immunol.* 24: 3233.

As with other chemokines, Tim-1 chemokine protein and variants exhibit binding activity with a number of chemokine receptors. Descriptions of chemokine-binding receptors and assays to detect such binding are described in Murphy *et al.*, *Science* 253: 1280 (1991); Holmes *et al.*, *Science* 253: 1278 (1991); Luster *et al.*, *J. Exp. Med.* 182: 219  
25 (1995); Loetscher *et al.*, *J. Biol. Chem.* 269: 232 (1994); Feng *et al.*, *Science* 272: 872 (1996); Neote *et al.*, *Cell* 72: 415 (1993); Gao *et al.*, *J. Exp. Med.* 177: 1421 (1993); Combadiere *et al.*, *J. Biol. Chem.* 270: 29671 (1995); Combadiere *et al.*, *J. Biol. Chem.* 270: 29671 (1995); Charo *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 2752 (1994); Ranci *et al.*, *J. Immunol.* 154: 6511 (1995); Post *et al.*, *J. Immunol.* 155: 5299 (1995); Daugherty *et al.*,



*J. Exp. Med.* 183: 2349 (1996); Power *et al.*, *J. Biol. Chem.* 270: 19495 (1995); Samson *et al.*, *Biochem.* 35: 3362 (1996); Raport *et al.*, *J. Biol. Chem.* 271:17161 (1996); Combadiere *et al.*, *J. Leukoc. Biol.* 60: 147 (1996); Baba *et al.*, *J. Biol. Chem.* 23: 14893 (1997); Yosida *et al.*, *J. Biol. Chem.* 272: 13803 (1997); Neote *et al.*, *J. Biol. Chem.* 268: 12247 (1993); Horuk *et al.*, *Science* 261: 1182 (1993); Ahuja *et al.*, *J. Biol. Chem.* 268: 28539 (1994); Gao *et al.*, *J. Biol. Chem.* 269: 28539 (1994); Arvanitakis *et al.*, *Nature* 385: 347 (1997).

Tim-1 proteins and variants can attract or activate a number of cell types including eosinophils, dendritic cells, basophils, and neutrophils. Assays for detecting eosinophil attraction are described in Dahinden *et al.*, *J. Exp. Med.* 179: 751 (1994), Weber *et al.*, *J. Immunol.* 154: 4166 (1995), and Noso *et al.*, *Biochem. Biophys. Res. Commun.* 200: 1470 (1994); for attracting dendritic cells, in Sozzani *et al.*, *J. Immunol.* 155: 3292 (1995); for attracting basophils, in Dahinden *et al.*, *J. Exp. Med.* 179: 751 (1994), Alam *et al.*, *J. Immunol.* 152: 1298 (1994), and Alam *et al.*, *J. Exp. Med.* 176: 781 (1992); and for activating neutrophils, in Maghazaci *et al.*, *Eur. J. Immunol.* 26: 315 (1996), and Taub *et al.*, *J. Immunol.* 155: 3877 (1995).

Tim-1 proteins and variants can act as mitogens for fibroblasts. An assay to test for this activity is described in Mullenbach *et al.*, *J. Biol. Chem.* 261: 719 (1986).

Tim-1 proteins and variants also exhibit kinase activation activity. Assays for such activity can be found, for example, in Yen *et al.*, *J. Leukoc. Biol.* 61: 529 (1997); Dubois *et al.*, *J. Immunol.* 156: 1356 (1996); and Turner *et al.*, *J. Immunol.* 155: 2437 (1995).

Tim-1 proteins and variants can act also as an inhibitors of angiogenesis or cell proliferation. Assays for such activities are described in Maione *et al.*, *Science* 247: 77 (1990).

Glycosaminoglycan production can be induced by Tim-1 proteins and variants. A method for detecting this activity is described in Castor *et al.*, *Proc. Natl. Acad. Sci. USA* 80: 765 (1983).

Histamine release from basophils is another activity of Tim-1 proteins and variants. Assays for histamine release are described in Dahinden *et al.*, *J. Exp. Med.* 170: 1787 (1989); and White *et al.*, *Immunol. Lett.* 22: 151 (1989).

5 Heparin binding is another activity that can be exhibited by Tim-1 proteins and variants. Means of measuring heparin binding are described in Luster *et al.*, *J. Exp. Med.* 182: 219 (1995).

In addition, Tim-1 proteins and variants can possess dimerization activity. Dimerization activity can be assayed according to Burrows *et al.*, *Biochem.* 33: 12741 (1994); and Zhang *et al.*, *Mol. Cell. Biol.* 15: 4851(1995), Dahinden *et al.*, *J. Exp. Med.* 10 170: 1787 (1989); and Feng *et al.*, *Science* 272: 872 (1996).

Tim-1 proteins and variants can play a role in the inflammatory response to viruses. This activity can be assayed as described in Bleul *et al.*, *Nature* 382: 829 (1996); and Oberlin *et al.*, *Nature* 382: 833 (1996).

Exocytosis of monocytes can be promoted by Tim-1 proteins and variants. An 15 assay for such activity is described in Ugucioni *et al.*, *Eur. J. Immunol.* 25: 64 (1995).

Tim-1 proteins and variants also can inhibit hematopoietic stem cell proliferation. A method for testing for such activity is taught in Graham *et al.*, *Nature* 344: 442 (1990).

Signal transduction assays can be used to screen Tim-1 proteins and variants for a desired level of biological activity. Generally, signal transduction activity occurs when 20 binding of a ligand to a receptor triggers a biological response in a cell or cell extract. The biological response is the result of a cascade of biochemical reactions. Measurement of any one of these reactions can indicate that the desired biological response was triggered.

Tim-1 proteins and variants bind to a class of receptors known as G-coupled proteins. When bound to a ligand, these receptors act on G-proteins coupled to the 25 receptors and can trigger, for example, an increase of intracellular  $Ca^{2+}$ ,  $IP_3$ , or DAG levels. Most cellular  $Ca^{2+}$  ions are sequestered in the mitochondria, endoplasmic reticulum, and other cytoplasmic vesicles, but binding of a Tim-1 protein or variant to a chemokine receptor can trigger the increase of free  $Ca^{2+}$  ions in the cytoplasm.

With fluorescent dyes, such as *fura-2*, the concentration of free  $\text{Ca}^{2+}$  can be monitored. An ester of *fura-2* is added to the media of host cells which express a Tim-1 receptor. The ester of *fura-2* is lipophilic and diffuses across the membrane. Once inside the cell, the *fura-2* ester is hydrolyzed by cytosolic esterases to its non-lipophilic form.

5 The dye cannot then diffuse out of the cell. The non-lipophilic form of *fura-2* will fluoresce when it binds to the free  $\text{Ca}^{2+}$  ions, which can be released after binding of a Tim-1 protein or variant to the receptor.

Fluorescence can be measured without lysing the cells at an excitation spectrum of 340 nM or 380 nM and at fluorescence spectrum of 500 nM. See Sakurai *et al.*, EP 480  
10 381, and Adachi *et al.*, *FEBS Lett* 311(2): 179-183 (1992), for examples of assays measuring free intracellular  $\text{Ca}^{2+}$  concentrations.

A rise in free cytosolic  $\text{Ca}^{2+}$  concentrations is preceded by the hydrolysis of phosphatidylinositol 4,5-bisphosphate. Hydrolysis of this phospholipid by the plasma-membrane enzyme phospholipase C yields 1,2-diacylglycerol (DAG), which remains in  
15 the membrane, and the water-soluble inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ). Binding of endothelin or endothelin agonists will increase the concentration of DAG and  $\text{IP}_3$ . Thus, signal transduction activity can be measured by monitoring the concentration of these hydrolysis products.

To measure the  $\text{IP}_3$  concentrations, radioactively labeled  $^3\text{H}$ -inositol is added to the  
20 media of host cells expressing a Tim-1 receptor. The  $^3\text{H}$ -inositol taken up by the cells. After stimulation of the cells with endothelin or endothelin agonist, the resulting inositol triphosphate is separated from the mono- and di-phosphate forms and measured. See Sakurai *et al.*, EP 480 381.

Alternatively, Amersham provides an inositol 1,4,5-trisphosphate assay system.  
25 With this system, Amersham provides tritylated inositol 1,4,5-trisphosphate and a receptor capable of distinguishing the radioactive inositol from other inositol phosphates. Routine competition assays can be performed to determine the inositol triphosphate levels using these reagents.

Tim-1 variants include glycosylated forms of Tim-1, aggregative conjugates of Tim-1 with other molecules, and covalent conjugates of Tim-1 with unrelated chemical moieties. Tim-1 variants also include allelic variants, species variants, and muteins. Truncations or deletions of regions which do not affect, for example, the inflammation-inducing activity of Tim-1 or the ability of Tim-1 to affect chemotaxis of neutrophils, lymphocytes, hemopoietic progenitors, monocytes, or natural killer cells are also Tim-1 variants. Covalent variants can be prepared by linking functionalities to groups which are found in the amino acid chain or at the N- or C-terminal residue, as is known in the art.

A subset of mutants, called muteins, is a group of polypeptides with the non-disulfide bond participating cysteines substituted with a neutral amino acid, generally, with serines. These mutants may be stable over a broader temperature range than Tim-1. See Mark *et al.*, U.S. Pat. No. 4,959,314. Tim-1 contains four cysteines that participate in disulfide bonding.

Tim-1 protein can be mutated to exhibit activities from related chemokines, such as IL-8. For example, amino acids 34, 35, and 36 of native Tim-1 can be mutated to glutamic acid, leucine, and arginine, respectively. This amino acid sequence, Glu-Leu-Arg, has been implicated in receptor binding of IL-8. Receptor binding can be assayed according to Murphy *et al.*, Science 253: 1280 (1991) and Holmes *et al.*, Science 253: 1278 (1991).

Mutants can also contain amino acid deletions or insertions compared to the native Tim-1. For example, deletion of the lysine at position 25 of native Tim-1 may be desirable to construct a mutant that exhibits properties of the CC chemokine subfamily, which include MIP-1 $\alpha$ .

Tim-1 can be extracted from Tim-1-producing human cells, such as brain, prostate, thymus, and peripheral blood lymphocytes using standard biochemical methods.

These methods include, but are not limited to, size exclusion chromatography, ammonium sulfate fractionation, ion exchange chromatography, affinity chromatography, crystallization, electrofocusing, and preparative gel electrophoresis. An isolated and purified Tim-1 protein or polypeptide is separated from other compounds which normally associate with a Tim-1 protein or polypeptide in a cell, such as certain proteins,

carbohydrates, lipids, or subcellular organelles. A preparation of isolated and purified Tim-1 proteins or polypeptides is at least 80% pure; preferably, the preparations are 90%, 95%, or 99% pure. Purity of a Tim-1 preparation can be assessed by any means known in the art, such as SDS-polyacrylamide gel electrophoresis.

5 Tim-1 proteins and polypeptides can also be produced by recombinant DNA methods or by synthetic chemical methods. For production of recombinant Tim-1 proteins or polypeptides, coding sequences selected from the *Tim-1* nucleotide sequence shown in SEQ ID NO:1, or variants of that sequence which encode Tim-1 protein, can be expressed in known prokaryotic or eukaryotic expression systems (see below). Bacterial, yeast,  
10 insect, or mammalian expression systems can be used, as is known in the art.

Alternatively, synthetic chemical methods, such as solid phase peptide synthesis, can be used to synthesize a Tim-1 protein or polypeptide. General means for the production of peptides, analogs or derivatives are outlined in CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS -- A SURVEY OF RECENT  
15 DEVELOPMENTS, Weinstein, B. ed., Marcell Dekker, Inc., publ., New York (1983). Moreover, substitution of D-amino acids for the normal L-stereoisomer can be carried out to increase the half-life of the molecule. Tim-1 variants can be similarly produced.

Fusion proteins comprising at least 6, 8, 10, 12, 15, 18, 20, 25, 50, 60, 75, 80, 90, or 100 or more contiguous Tim-1 amino acids can also be constructed. Human Tim-1  
20 fusion proteins are useful for generating antibodies against Tim-1 amino acid sequences and for use in various assay systems. For example, Tim-1 fusion proteins can be used to identify proteins which interact with Tim-1 protein and influence any of the functions of Tim-1 described above. Physical methods, such as protein affinity chromatography, or library-based assays for protein-protein interactions, such as the yeast two-hybrid or phage  
25 display systems, can also be used for this purpose. Such methods are well known in the art and can also be used as drug screens.

A Tim-1 fusion protein comprises two protein segments fused together by means of a peptide bond. The first protein segment comprises at least 6, 8, 10, 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 75, 80, 90, or 100 or more contiguous amino acids of a Tim-1 protein.

The amino acids can be selected from the amino acid sequence shown in SEQ ID NO:2 or from a biologically active variant of that sequence, such as those described above. The first protein segment can also comprise full-length Tim-1.

5 The second protein segment can be a full-length protein or a protein fragment or polypeptide. Proteins commonly used in fusion protein construction include  $\beta$ -galactosidase,  $\beta$ -glucuronidase, green fluorescent protein (GFP), autofluorescent proteins, including blue fluorescent protein (BFP), glutathione-S-transferase (GST), luciferase, horseradish peroxidase (HRP), and chloramphenicol acetyltransferase (CAT). Epitope  
10 tags can be used in fusion protein constructions, including histidine (His) tags, FLAG tags, influenza hemagglutinin (HA) tags, Myc tags, VSV-G tags, and thioredoxin (Trx) tags. Other fusion constructions can include maltose binding protein (MBP), S-tag, Lex A DNA binding domain (DID) fusions, GAL4 DNA binding domain fusions, and herpes simplex virus (HSV) BP16 protein fusions.

Tim-1 fusion proteins can be made by covalently linking the first and second two  
15 protein segments or by standard procedures in the art of molecular biology. Recombinant DNA methods can be used to prepare Tim-1 fusion proteins, for example, by making a DNA construct which comprises coding sequences selected from SEQ ID NO:1 in proper reading frame with nucleotides encoding the second protein segment and expressing the DNA construct in a host cell, as is known in the art. Many kits for constructing fusion  
20 proteins are available from companies which supply research labs with tools for experiments, including, for example, Promega Corporation (Madison, WI), Stratagene (La Jolla, CA), Clontech (Mountain View, CA), Santa Cruz Biotechnology (Santa Cruz, CA), MBL International Corporation (MIC; Watertown, MA), and Quantum Biotechnologies (Montreal, Canada; 1-888-DNA-KITS).

25 Isolated and purified Tim-1 proteins, polypeptides, variants, or fusion proteins can be used as immunogens, to obtain preparations of antibodies which specifically bind to Tim-1 protein. The antibodies can be used, *inter alia*, to detect wild-type Tim-1 protein in human tissue and fractions thereof. The antibodies can also be used to detect the presence

of mutations in the *Tim-1* gene which result in under- or over-expression of a Tim-1 protein or in expression of a Tim-1 protein with altered size or electrophoretic mobility.

Antibodies which specifically bind to epitopes of Tim-1 proteins, polypeptides, fusion proteins, or biologically active variants can be used in immunochemical assays, including but not limited to Western blots, ELISAs, radioimmunoassays, immunohistochemical assays, immunoprecipitations, or other immunochemical assays known in the art. Typically, antibodies of the invention provide a detection signal at least 5-, 10-, or 20-fold higher than a detection signal provided with other proteins when used in such immunochemical assays. Preferably, antibodies which specifically bind to Tim-1 epitopes do not detect other proteins in immunochemical assays and can immunoprecipitate Tim-1 protein or polypeptides from solution.

Tim-1-specific antibodies specifically bind to epitopes present in a Tim-1 protein having the amino acid sequence shown in SEQ ID NO:2 or to biologically active variants of that sequence. Typically, at least 6, 8, 10, or 12 contiguous amino acids are required to form an epitope. However, epitopes which involve non-contiguous amino acids may require more, *e.g.*, at least 15, 25, or 50 amino acids. Preferably, Tim-1 epitopes are not present in other human proteins.

Epitopes of Tim-1 which are particularly antigenic can be selected, for example, by routine screening of Tim-1 polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein to the amino acid sequence shown in SEQ ID NO:2. Such methods are taught, for example, in Hopp and Wood, *Proc. Natl. Acad. Sci. U.S.A.* 78, 3824-28 (1981), Hopp and Wood, *Mol. Immunol.* 20, 483-89 (1983), and Sutcliffe *et al.*, *Science* 219, 660-66 (1983).

Any type of antibody known in the art can be generated to bind specifically to Tim-1 epitopes. For example, preparations of polyclonal and monoclonal antibodies can be made using standard methods which are well known in the art. Similarly, single-chain antibodies can also be prepared. Single-chain antibodies which specifically bind to Tim-1 epitopes can be isolated, for example, from single-chain immunoglobulin display libraries, as is known in the art. The library is "panned" against Tim-1 amino acid sequences, and a

number of single chain antibodies which bind with high-affinity to different epitopes of Tim-1 protein can be isolated. Hayashi *et al.*, 1995, *Gene* 160:129-30. Single-chain antibodies can also be constructed using a DNA amplification method, such as the polymerase chain reaction (PCR), using hybridoma cDNA as a template. Thirion *et al.*,  
5 1996, *Eur. J. Cancer Prev.* 5:507-11.

Single-chain antibodies can be mono- or bispecific, and can be bivalent or tetravalent. Construction of tetravalent, bispecific single-chain antibodies is taught, for example, in Coloma and Morrison, 1997, *Nat. Biotechnol.* 15:159-63. Construction of bivalent, bispecific single-chain antibodies is taught *inter alia* in Mallender and Voss,  
10 1994, *J. Biol. Chem.* 269:199-206.

A nucleotide sequence encoding a single-chain antibody can be constructed using manual or automated nucleotide synthesis, cloned into an expression construct using standard recombinant DNA methods, and introduced into a cell to express the coding sequence, as described below. Alternatively, single-chain antibodies can be produced  
15 directly using, for example, filamentous phage technology. Verhaar *et al.*, 1995, *Int. J. Cancer* 61:497-501; Nicholls *et al.*, 1993, *J. Immunol. Meth.* 165:81-91.

Monoclonal and other antibodies can also be "humanized" in order to prevent a patient from mounting an immune response against the antibody when it is used therapeutically. Such antibodies may be sufficiently similar in sequence to human  
20 antibodies to be used directly in therapy or may require alteration of a few key residues. Sequence differences between, for example, rodent antibodies and human sequences can be minimized by replacing residues which differ from those in the human sequences, for example, by site directed mutagenesis of individual residues, or by grafting of entire complementarity determining regions. Alternatively, one can produce humanized  
25 antibodies using recombinant methods, as described in GB2188638B. Antibodies which specifically bind to Tim-1 epitopes can contain antigen binding sites which are either partially or fully humanized, as disclosed in U.S. 5,565,332.

Other types of antibodies can be constructed and used therapeutically in methods of the invention. For example, chimeric antibodies can be constructed as disclosed, for



example, in WO 93/03151. Binding proteins which are derived from immunoglobulins and which are multivalent and multispecific, such as the "diabodies" described in WO 94/13804, can also be prepared.

Antibodies of the invention can be purified by methods well known in the art. For example, antibodies can be affinity purified by passing the antibodies over a column to which a Tim-1 protein, polypeptide, biologically active variant, or fusion protein is bound. The bound antibodies can then be eluted from the column, using a buffer with a high salt concentration.

Tim-1-specific binding polypeptides other than antibodies can also be generated. Tim-1-specific binding polypeptides are polypeptides which bind with Tim-1 or its variants and which have a measurably higher binding affinity for the Tim-1 and polypeptide derivatives of Tim-1 than for other polypeptides tested for binding. Higher affinity by a factor of 10 is preferred, more preferably a factor of 100. Such polypeptides can be found, for example, using the yeast two-hybrid system.

The coding region of the *Tim-1* gene has the nucleotide sequence shown in SEQ ID NO:1. Isolated *Tim-1* polynucleotides according to the invention are subgenomic contain less than a whole chromosome. Preferably, the subgenomic polynucleotides are intron-free.

Isolated *Tim-1* subgenomic polynucleotides of the invention can comprise at least 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 75, 100, 125, 150, 154, 175, or 200 or more contiguous nucleotides selected from SEQ ID NO:1 or call comprise SEQ ID NO:1. Such subgenomic polynucleotides can be used, for example, as primers or probes or for expression of Tim-1 proteins or polypeptides.

The complement of the nucleotide sequence shown in SEQ ID NO:1 is a contiguous nucleotide sequence which forms Watson-Crick base pairs with a contiguous nucleotide sequence shown in SEQ ID NO:1. The complement of SEQ ID NO:1 is a subgenomic polynucleotide of the invention and can be used, for example, to provide *Tim-1* antisense oligonucleotides, primers, and probes.

Antisense oligonucleotides and probes of the invention can consist of at least 11, 12, 15, 20, 25, 30, 50, or 100 contiguous nucleotides which are complementary to the coding sequence shown in SEQ ID NO:1. A complement of the entire coding sequence can also be used. Double-stranded subgenomic polynucleotides which comprise all or a portion of the nucleotide sequence shown in SEQ ID NO:1, as well as subgenomic polynucleotides which encode Tim-1-specific antibodies or ribozymes, are also subgenomic polynucleotides of the invention.

Degenerate nucleotide sequences encoding amino acid sequences of Tim-1 protein or biologically active Tim-1 variants, as well as homologous nucleotide sequences which are at least 65%, 75%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleotide sequence shown in SEQ ID NO:1, are also *Tim-1* subgenomic polynucleotides. Percent sequence identity between the nucleotide sequence of SEQ ID NO:1 and a putative homologous or degenerate *Tim-1* nucleotide sequence is determined using computer programs which employ the Smith-Waterman algorithm, for example as implemented in the MPSRCH program (Oxford Molecular), using an affine gap search with the following parameters: a gap open penalty of 12 and a gap extension penalty of 1.

Nucleotide sequences which hybridize to the coding sequence shown in SEQ ID NO:1 or its complement with at most 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, or 35% basepair mismatches are also *Tim-1* subgenomic polynucleotides of the invention. For example, by using the following wash conditions--2X SCC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, room temperature twice, 30 minutes each; then 2X SCC, 0.1% SDS, 50 °C once, 30 minutes; then 2X SCC, room temperature twice, 10 minutes each--homologous *Tim-1* sequences can be identified which contain at most about 25-30% basepair mismatches with SEQ ID NO:1 or its complement. More preferably, homologous nucleic acid strands contain 15-25% basepair mismatches, even more preferably 5-15% basepair mismatches.

Nucleotide sequences which hybridize to the coding sequence shown in SEQ ID NO:1 or its complement following stringent hybridization and/or wash conditions are also *Tim-1* subgenomic polynucleotides of the invention. Stringent wash conditions are well

known and understood in the art and are disclosed, for example, in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed., 1989, at pages 9.50-9.51.

Typically, for stringent hybridization conditions a combination of temperature and salt concentration should be chosen that is approximately 12-20 °C below the calculated

5  $T_m$  of the hybrid under study. It is well known that the  $T_m$  of a double-stranded DNA decreases by 1-1.5 °C with every 1% decrease in homology (Bonner *et al.*, *J. Mol. Biol.* 81, 123 (1973). The  $T_m$  of a hybrid between the *Tim-1* sequence shown in SEQ ID NO:1 and a polynucleotide sequence which is 65%, 75%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO:1 can be calculated, for example, using the equation of

10 Bolton and McCarthy, *Proc. Natl. Acad. Sci. U.S.A.* 48, 1390 (1962):

$$T_m = 81.5\text{ }^{\circ}\text{C} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%G + C) - 0.63(\%\text{formamide}) - 600/l,$$

where  $l$  = the length of the hybrid in basepairs.

Stringent wash conditions include, for example, 4X SSC at 65 °C, or 50% formamide, 4X SSC at 42 °C, or 0.5X SSC, 0.1% SDS at 65 °C. Highly stringent wash conditions include,

15 for example, 0.2X SSC at 65 °C.

*Tim-1* subgenomic polynucleotides can be isolated and purified free from other nucleotide sequences using standard nucleic acid purification techniques. For example, restriction enzymes and probes can be used to isolate subgenomic polynucleotide fragments which comprise the *Tim-1* coding sequences. Isolated and purified *Tim-1* subgenomic polynucleotides are in preparations which are free or at least 90% free of other molecules.

Complementary DNA (cDNA) molecules which encode *Tim-1* proteins are also *Tim-1* subgenomic polynucleotides of the invention. *Tim-1* cDNA molecules can be made with standard molecular biology techniques, using *Tim-1* mRNA as a template. *Tim-1* cDNA molecules can thereafter be replicated using molecular biology techniques known in the art and disclosed in manuals such as Sambrook *et al.*, 1989. An amplification technique, such as the polymerase chain reaction (PCR), can be used to obtain additional copies of subgenomic polynucleotides of the invention, using either human genomic DNA or cDNA as a template.

Alternatively, synthetic chemistry techniques can be used to synthesize *Tim-1* subgenomic polynucleotide molecules of the invention. The degeneracy of the genetic code allows alternate nucleotide sequences to be synthesized which will encode a Tim-1 protein having the amino acid sequence shown in SEQ ID NO:2 or a biologically active variant of that sequence. All such nucleotide sequences are within the scope of the present invention.

The invention also provides polynucleotide probes which can be used to detect *Tim-1* sequences, for example, in hybridization protocols such as Northern or Southern blotting or *in situ* hybridizations. Polynucleotide probes of the invention comprise at least 12, 13, 14, 15, 16, 17, 18, 19, 20, 30, or 40 or more contiguous nucleotides selected from SEQ ID NO:1. Polynucleotide probes of the invention can comprise a detectable label, such as a radioisotopic, fluorescent, enzymatic, or chemiluminescent label.

A *Tim-1* subgenomic polynucleotide comprising *Tim-1* coding sequences can be used in a construct, such as a DNA or RNA construct. *Tim-1* subgenomic polynucleotides can be propagated in vectors and cell lines using techniques well known in the art. *Tim-1* subgenomic polynucleotides can be on linear or circular molecules. They can be on autonomously replicating molecules or on molecules without replication sequences. They can be regulated by their own or by other regulatory sequences, as are known in the art.

A host cell comprising a *Tim-1* expression construct can be used to express all or a portion of a Tim-1 protein. Host cells comprising *Tim-1* expression constructs can be prokaryotic or eukaryotic. A variety of host cells are available for use in bacterial, yeast, insect, and human expression systems and can be used to express or to propagate *Tim-1* expression constructs (see below). Expression constructs can be introduced into host cells using any technique known in the art. These techniques include transferrin-polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated cellular fusion, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, and calcium phosphate-mediated transfection.

A *Tim-1* expression construct comprises a promoter which is functional in a chosen host cell. The skilled artisan can readily select an appropriate promoter from the

large number of cell type-specific promoters known and used in the art. The expression construct can also contain a transcription terminator which is functional in the host cell. The expression construct comprises a polynucleotide segment which encodes all or a portion of the Tim-1 protein, variant, fusion protein, antibody, or ribozyme. The polynucleotide segment is located downstream from the promoter. Transcription of the polynucleotide segment initiates at the promoter. The expression construct can be linear or circular and can contain sequences, if desired, for autonomous replication.

Bacterial systems for expressing *Tim-1* expression constructs include those described in Chang *et al.*, *Nature* (1978) 275: 615, Goeddel *et al.*, *Nature* (1979) 281: 544, Goeddel *et al.*, *Nucleic Acids Res.* (1980) 8: 4057, EP 36,776, U.S. 4,551,433, deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* (1983) 80: 21-25, and Siebenlist *et al.*, *Cell* (1980) 20: 269.

Expression systems in yeast include those described in Hinnen *et al.*, *Proc. Natl. Acad. Sci. USA* (1978) 75: 1929; Ito *et al.*, *J. Bacteriol.* (1983) 153: 163; Kurtz *et al.*, *Mol. Cell. Biol.* (1986) 6: 142; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; Gleeson *et al.*, *J. Gen. Microbiol.* (1986) 132: 3459, Roggenkamp *et al.*, *Mol. Gen. Genet.* (1986) 202 :302) Das *et al.*, *J. Bacteriol.* (1984) 158: 1165; De Louvencourt *et al.*, *J. Bacteriol.* (1983) 154: 737, Van den Berg *et al.*, *Bio/Technology* (1990) 8: 135; Kunze *et al.*, *J. Basic Microbiol.* (1985) 25: 141; Cregg *et al.*, *Mol. Cell. Biol.* (1985) 5: 3376, U.S. 4,837,148, US 4,929,555; Beach and Nurse, *Nature* (1981) 300: 706; Davidow *et al.*, *Curr. Genet.* (1985) 10: 380, Gaillardin *et al.*, *Curr. Genet.* (1985) 10: 49, Ballance *et al.*, *Biochem. Biophys. Res. Commun.* (1983) 112: 284-289; Tilburn *et al.*, *Gene* (1983) 26: 205-221, Yelton *et al.*, *Proc. Natl. Acad. Sci. USA* (1984) 81: 1470-1474, Kelly and Hynes, *EMBO J.* (1985) 4: 475479; EP 244,234, and WO 91/00357.

Expression of *Tim-1* expression constructs in insects can be carried out as described in U.S. 4,745,051, Friesen *et al.* (1986) "The Regulation of Baculovirus Gene Expression" in: THE MOLECULAR BIOLOGY OF BACULOVIRUSES (W. Doerfler, ed.), EP 127,839, EP 155,476, and Vlak *et al.*, *J. Gen. Virol.* (1988) 69: 765-776, Miller *et al.*, *Ann. Rev. Microbiol.* (1988) 42: 177, Carbonell *et al.*, *Gene* (1988) 73: 409, Maeda *et al.*, *Nature* (1985) 315: 592-594, Lebacqz-Verheyden *et al.*, *Mol. Cell. Biol.* (1988) 8: 3129;

Smith *et al.*, *Proc. Natl. Acad. Sci. USA* (1985) 82: 8404, Miyajima *et al.*, *Gene* (1987) 58: 273; and Martin *et al.*, *DNA* (1988) 7:99. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts are described in Luckow *et al.*, *Bio/Technology* (1988) 6: 47-55, Miller *et al.*, in GENETIC ENGINEERING (Setlow, J.K. *et al.* eds.), Vol. 8 (Plenum Publishing, 1986), pp. 277-279, and Maeda *et al.*, *Nature*, (1985) 315: 592-594.

Mammalian expression of *Tim-1* expression constructs can be achieved as described in Dijkema *et al.*, *EMBO J.* (1985) 4: 761, Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* (1982b) 79: 6777, Boshart *et al.*, *Cell* (1985) 41: 521 and U.S. 4,399,216. Other features of mammalian expression of *Tim-1* expression constructs can be facilitated as described in Ham and Wallace, *Meth. Enz.* (1979) 58: 44, Barnes and Sato, *Anal. Biochem.* (1980) 102: 255, U.S. 4,767,704, US 4,657,866, US 4,927,762, US 4,560,655, WO 90/103430, WO 87/00195, and U.S. RE 30,985.

Subgenomic polynucleotides of the invention can also be used in gene delivery vehicles, for the purpose of delivering a *Tim-1* mRNA or oligonucleotide (either with the sequence of native *Tim-1* mRNA or its complement), full-length Tim-1 protein, Tim-1 fusion protein, Tim-1 polypeptide, or Tim-1-specific ribozyme or single-chain antibody, into a cell preferably a eukaryotic cell. According to the present invention, a gene delivery vehicle can be, for example, naked plasmid DNA, a viral expression vector comprising a *Tim-1* subgenomic polynucleotide, or a *Tim-1* subgenomic polynucleotide in conjunction with a liposome or a condensing agent.

In one embodiment of the invention, the gene delivery vehicle comprises a promoter and a *Tim-1* subgenomic polynucleotide. Preferred promoters are tissue-specific promoters and promoters which are activated by cellular proliferation, such as the thymidine kinase and thymidylate synthase promoters. Other preferred promoters include promoters which are activatable by infection with a virus, such as the  $\alpha$ - and  $\beta$ -interferon promoters, and promoters which are activatable by a hormone, such as estrogen. Other promoters which can be used include the Moloney virus LTR, the CMV promoter, and the mouse albumin promoter.

A *Tim-1* gene delivery vehicle can comprise viral sequences such as a viral origin of replication or packaging signal. These viral sequences can be selected from viruses such as astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the *Tim-1* gene delivery vehicle is a recombinant retroviral vector. Recombinant retroviruses and various uses thereof have been described in numerous references including, for example, Mann *et al.*, *Cell* 33:153, 1983, Cane and Mulligan, *Proc. Nat'l. Acad. Sci. USA* 81:6349, 1984, Miller *et al.*, *Human Gene Therapy* 1:5-14, 1990, U.S. Patent Nos. 4,405,712, 4,861,719, and 4,980,289, and PCT Application Nos. WO 89/02,468, WO 89/05,349, and WO 90/02,806. Numerous retroviral gene delivery vehicles can be utilized in the present invention, including for example those described in EP 0,415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res.* 53:3860-3864, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram *et al.*, *Cancer Res.* 53:83-88, 1993; Takamiya *et al.*, *J. Neurosci. Res.* 33:493-503, 1992; Baba *et al.*, *J. Neurosurg.* 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805).

Particularly preferred retroviruses are derived from retroviruses which include avian leukosis virus (ATCC Nos. VR-535 and VR-247), bovine leukemia virus (VR-1315), murine leukemia virus (MLV), mink-cell focus-inducing virus (Koch *et al.*, *J. Vir.* 49:828, 1984; and Oliff *et al.*, *J. Vir.* 48:542, 1983), murine sarcoma virus (ATCC Nos. VR-844, 45010 and 45016), reticuloendotheliosis virus (ATCC Nos VR-994, VR-770 and 45011), Rous sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus, endogenous feline retrovirus (*e.g.*, RD114), and mouse or rat gL30 sequences used as a retroviral vector. Particularly preferred strains of MLV from which recombinant retroviruses can be generated include 4070A and 1504A (Hartley and Rowe, *J. Vir.* 19:19, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi (Ru *et al.*, *J. Vir.* 67:4722, 1993; and Yantchev *Neoplasma* 26:397, 1979), Gross (ATCC No. VR-590), Kirsten (Albino *et al.*, *J. Exp. Med.* 164:1710, 1986), Harvey sarcoma virus (Manly *et al.*,

*J. Vir.* 62:3540, 1988; and Albino *et al.*, *J. Exp. Med.* 164:1710, 1986) and Rauscher (ATCC No. VR-998), and Moloney MLV (ATCC No. VR-190). A particularly preferred non-mouse retrovirus is Rous sarcoma virus. Preferred Rous sarcoma viruses include Bratislava (Manly *et al.*, *J. Vir.* 62:3540, 1988; and Albino *et al.*, *J. Exp. Med.* 164:1710, 1986), Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard (ATCC No. VR-140), Carr-Zilber (Adgighitov *et al.*, *Neoplasma* 27:159, 1980), Engelbreth-Holm (Laurent *et al.*, *Biochem Biophys Acta* 908:241, 1987), Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354) viruses.

Any of the above retroviruses can be readily utilized in order to assemble or construct retroviral *Tim-1* gene delivery vehicles given the disclosure provided herein and standard recombinant techniques (*e.g.*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989, and Kunkle, *PROC. NATL. ACAD. SCI. U.S.A.* 82:488, 1985) known in the art. Portions of retroviral *Tim-1* expression vectors can be derived from different retroviruses. For example, retrovector LTRs can be derived from a murine sarcoma virus, a tRNA binding site from a Rous sarcoma virus, a packaging signal from a murine leukemia virus, and an origin of second strand synthesis from an avian leukosis virus. These recombinant retroviral vectors can be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (*see* Serial No. 07/800,921, filed November 29, 1991). Recombinant retroviruses can be produced which direct the site-specific integration of the recombinant retroviral genome into specific regions of the host cell DNA. Such site-specific integration can be mediated by a chimeric integrase incorporated into the retroviral particle (*see* Serial No. 08/445,466 filed May 22, 1995). It is preferable that the recombinant viral gene delivery vehicle is a replication-defective recombinant virus.

Packaging cell lines suitable for use with the above-described retroviral gene delivery vehicles can be readily prepared (*see* Serial No. 08/240,030, filed May 9, 1994; *see also* WO 92/05266) and used to create producer cell lines (also termed vector cell lines



or "VCLs") for production of recombinant viral particles. In particularly preferred embodiments of the present invention, packaging cell lines are made from human (e.g., HT1080 cells) or mink parent cell lines, thereby allowing production of recombinant retroviral gene delivery vehicles which are capable of surviving inactivation in human serum. The construction of recombinant retroviral gene delivery vehicles is described in detail in WO 91/02805. These recombinant retroviral gene delivery vehicles can be used to generate transduction competent retroviral particles by introducing them into appropriate packaging cell lines (*see* Serial No. 07/800,921). Similarly, adenovirus gene delivery vehicles can also be readily prepared and utilized given the disclosure provided herein (*see also* Berkner, *Biotechniques* 6:616-627, 1988, and Rosenfeld *et al.*, *Science* 252:431-434, 1991, WO 93/07283, WO 93/06223, and WO 93/07282).

A *Tim-1* gene delivery vehicle can also be a recombinant adenoviral gene delivery vehicle. Such vehicles can be readily prepared and utilized given the disclosure provided herein (*see* Berkner, *Biotechniques* 6:616, 1988, and Rosenfeld *et al.*, *Science* 252:431, 1991, WO 93/07283, WO 93/06223, and WO 93/07282). Adeno-associated viral *Tim-1* gene delivery vehicles can also be constructed and used to deliver *Tim-1* amino acids or nucleotides. The use of adeno-associated viral gene delivery vehicles *in vitro* is described in Chatterjee *et al.*, *Science* 258: 1485-1488 (1992), Walsh *et al.*, *Proc. Nat'l. Acad. Sci.* 89: 7257-7261 (1992), Walsh *et al.*, *J. Clin. Invest.* 94: 1440-1448 (1994), Flotte *et al.*, *J. Biol. Chem.* 268: 3781-3790 (1993), Ponnazhagan *et al.*, *J. Exp. Med.* 179: 733-738 (1994), Miller *et al.*, *Proc. Nat'l Acad. Sci.* 91: 10183-10187 (1994), Einerhand *et al.*, *Gene Ther.* 2: 336-343 (1995), Luo *et al.*, *Exp. Hematol.* 23: 1261-1267 (1995), and Zhou *et al.*, *Gene Therapy* 3: 223-229 (1996). *In vivo* use of these vehicles is described in Flotte *et al.*, *Proc. Nat'l Acad. Sci.* 90: 10613-10617 (1993), and Kaplitt *et al.*, *Nature Genet.* 8:148-153 (1994).

In another embodiment of the invention, a *Tim-1* gene delivery vehicle is derived from a togavirus. Preferred togaviruses include alphaviruses, in particular those described in U.S. Serial No. 08/405,627, filed March 15, 1995, WO 95/07994. Alpha viruses, including Sindbis and ELVS viruses can be gene delivery vehicles for *Tim-1*

polynucleotides. Alpha viruses are described in WO 94/21792, WO 92/10578 and WO 95/07994. Several different alphavirus gene delivery vehicle systems can be constructed and used to deliver *Tim-1* subgenomic polynucleotides to a cell according to the present invention. Representative examples of such systems include those described in U.S.

- 5 Patents 5,091,309 and 5,217,879. Particularly preferred alphavirus gene delivery vehicles for use in the present invention include those which are described in WO 95/07994, and U.S. Serial No. 08/405,627.

Preferably, the recombinant viral vehicle is a recombinant alphavirus viral vehicle based on a Sindbis virus. Sindbis constructs, as well as numerous similar constructs, can  
10 be readily prepared essentially as described in U.S. Serial No. 08/198,450. Sindbis viral gene delivery vehicles typically comprise a 5' sequence capable of initiating Sindbis virus transcription, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region inactivated so as to prevent subgenomic fragment transcription, and a Sindbis RNA polymerase recognition sequence. Optionally, the viral junction region can  
15 be modified so that subgenomic polynucleotide transcription is reduced, increased, or maintained. As will be appreciated by those in the art, corresponding regions from other alphaviruses can be used in place of those described above.

The viral junction region of an alphavirus-derived gene delivery vehicle can comprise a first viral junction region which has been inactivated in order to prevent  
20 transcription of the subgenomic polynucleotide and a second viral junction region which has been modified such that subgenomic polynucleotide transcription is reduced. An alphavirus-derived vehicle can also include a 5' promoter capable of initiating synthesis of viral RNA from cDNA and a 3' sequence which controls transcription termination.

Other recombinant togaviral gene delivery vehicles which can be utilized in the  
25 present invention include those derived from Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described in U.S. Patents 5,091,309 and 5,217,879

and in WO 92/10578. The Sindbis vehicles described above, as well as numerous similar constructs, can be readily prepared essentially as described in U.S. Serial No. 08/198,450.

Other viral gene delivery vehicles suitable for use in the present invention include, for example, those derived from poliovirus (Evans *et al.*, *Nature* 339:385, 1989, and Sabin *et al.*, *J. Biol. Standardization* 1:115, 1973) (ATCC VR-58); rhinovirus (Arnold *et al.*, *J. Cell. Biochem.* L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *PROC. NATL. ACAD. SCI. U.S.A.* 86:317, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci.* 569:86, 1989; Flexner *et al.*, *Vaccine* 8:17, 1990; U.S. 4,603,112 and U.S. 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan *et al.*, *Nature* 277:108, 1979) (ATCC VR-305), (Madzak *et al.*, *J. Gen. Vir.* 73:1533, 1992); influenza virus (Luytjes *et al.*, *Cell* 59:1107, 1989; McMichael *et al.*, *The New England Journal of Medicine* 309:13, 1983; and Yap *et al.*, *Nature* 273:238, 1978) (ATCC VR-797); parvovirus such as adeno-associated virus (Samulski *et al.*, *J. Vir.* 63:3822, 1989, and Mendelson *et al.*, *Virology* 166:154, 1988) (ATCC VR-645); herpes simplex virus (Kit *et al.*, *Adv. Exp. Med. Biol.* 215:219, 1989) (ATCC VR-977; ATCC VR-260); *Nature* 277: 108, 1979); human immunodeficiency virus (EPO 386,882, Buchschacher *et al.*, *J. Vir.* 66:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); A (ATCC VR-67; ATCC VR-1247), Aura (ATCC VR-368), Bebaru virus (ATCC VR-600; ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64; ATCC VR-1241), Fort Morgan (ATCC VR-924), Getah virus (ATCC VR-369; ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mucambo virus (ATCC VR-580; ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372; ATCC VR-1245), Tonate (ATCC VR-925), Trinita (ATCC VR-469), Una (ATCC VR-374), Whataroa (ATCC VR-926), Y-62-33 (ATCC VR-375), O'Nyong virus, Eastern encephalitis virus (ATCC VR-65; ATCC VR-1242), Western encephalitis virus (ATCC VR-70; ATCC VR-1251; ATCC VR-622; ATCC VR-1252), and coronavirus (Hamre *et al.*, *Proc. Soc. Exp. Biol. Med.* 121:190, 1966) (ATCC VR-740).

A subgenomic *Tim-1* polynucleotide of the invention can also be combined with a condensing agent to form a gene delivery vehicle. In a preferred embodiment, the

condensing agent is a polycation, such as polylysine, polyarginine, polyornithine, protamine, spermine, spermidine, and putrescine. Many suitable methods for making such linkages are known in the art (see, for example, Serial No. 08/366,787, filed December 30, 1994).

5 In an alternative embodiment, a *Tim-1* subgenomic polynucleotide is associated with a liposome to form a gene delivery vehicle. Liposomes are small, lipid vesicles comprised of an aqueous compartment enclosed by a lipid bilayer, typically spherical or slightly elongated structures several hundred Angstroms in diameter. Under appropriate conditions, a liposome can fuse with the plasma membrane of a cell or with the membrane  
10 of an endocytic vesicle within a cell which has internalized the liposome, thereby releasing its contents into the cytoplasm. Prior to interaction with the surface of a cell, however, the liposome membrane acts as a relatively impermeable barrier which sequesters and protects its contents, for example, from degradative enzymes. Additionally, because a liposome is a synthetic structure, specially designed liposomes can be produced which incorporate  
15 desirable features. See Stryer, *Biochemistry*, pp. 236-240, 1975 (W.H. Freeman, San Francisco, CA); Szoka *et al.*, *Biochim. Biophys. Acta* 600:1, 1980; Bayer *et al.*, *Biochim. Biophys. Acta* 550:464, 1979; Rivnay *et al.*, *Meth. Enzymol.* 149:119, 1987; Wang *et al.*, *PROC. NATL. ACAD. SCI. U.S.A.* 84: 7851, 1987, Plant *et al.*, *Anal. Biochem.* 176:420, 1989, and U.S. Patent 4,762,915. Liposomes can encapsulate a variety of nucleic acid  
20 molecules including DNA, RNA, plasmids, and expression constructs comprising *Tim-1* subgenomic polynucleotides such those disclosed in the present invention.

Liposomal preparations for use in the present invention include cationic (positively charged), anionic (negatively charged) and neutral preparations. Cationic liposomes have been shown to mediate intracellular delivery of plasmid DNA (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7416, 1987), mRNA (Malone *et al.*, *Proc. Natl. Acad. Sci. USA*  
25 86:6077-6081, 1989), and purified transcription factors (Debs *et al.*, *J. Biol. Chem.* 265:10189-10192, 1990), in functional form. Cationic liposomes are readily available. For example, N[1-2,3-dioleoyloxy)propyl]-N,N,N-triethylammonium (DOTMA) liposomes are available under the trademark Lipofectin, from GIBCO BRL, Grand Island, NY. See

also Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 5148-5152.87, 1994. Other commercially available liposomes include Transfectace (DDAB/DOPE) and DOTAP/DOPE (Boehringer). Other cationic liposomes can be prepared from readily available materials using techniques well known in the art. See, e.g., Szoka *et al.*, *Proc.*  
5 *Natl. Acad. Sci. USA* 75:4194-4198, 1978; and WO 90/11092 for descriptions of the synthesis of DOTAP (1,2-bis(oleoyloxy)-3-(trimethylammonio)propane) liposomes.

Similarly, anionic and neutral liposomes are readily available, such as from Avanti Polar Lipids (Birmingham, AL), or can be easily prepared using readily available materials. Such materials include phosphatidyl choline, cholesterol, phosphatidyl  
10 ethanolamine, dioleoylphosphatidyl choline (DOPC), dioleoylphosphatidyl glycerol (DOPG), dioleoylphosphatidyl ethanolamine (DOPE), among others. These materials can also be mixed with the DOTMA and DOTAP starting materials in appropriate ratios. Methods for making liposomes using these materials are well known in the art.

The liposomes can comprise multilamellar vesicles (MLVs), small unilamellar  
15 vesicles (SUVs), or large unilamellar vesicles (LUVs). The various liposome-nucleic acid complexes are prepared using methods known in the art. See, e.g., Straubinger *et al.*, *METHODS OF IMMUNOLOGY* (1983), Vol. 101, pp. 512-527; Szoka *et al.*, *Proc. Natl. Acad. Sci. USA* 87:3410-3414, 1990; Papahadjopoulos *et al.*, *Biochim. Biophys. Acta* 394:483, 1975; Wilson *et al.*, *Cell* 17:77, 1979; Deamer and Bangham, *Biochim. Biophys. Acta*  
20 443:629, 1976; Ostro *et al.*, *Biochem. Biophys. Res. Commun.* 76:836, 1977; Fraley *et al.*, *Proc. Natl. Acad. Sci. USA* 76:3348, 1979; Enoch and Strittmatter, *Proc. Natl. Acad. Sci. USA* 76:145, 1979; Fraley *et al.*, *J. Biol. Chem.* 255:10431, 1980; Szoka and Papahadjopoulos, *Proc. Natl. Acad. Sci. USA* 75:145, 1979; and Schaefer-Ridder *et al.*, *Science* 215:166, 1982.

25 In addition, lipoproteins can be included with a *Tim-1* subgenomic polynucleotide for delivery to a cell. Examples of such lipoproteins include chylomicrons, HDL, IDL, LDL, and VLDL. Mutants, fragments, or fusions of these proteins can also be used. Modifications of naturally occurring lipoproteins can also be used, such as acetylated LDL. These lipoproteins can target the delivery of polynucleotides to cells expressing

lipoprotein receptors. Preferably, if lipoproteins are included with a polynucleotide, no other targeting ligand is included in the composition.

In another embodiment, naked *Tim-1* subgenomic polynucleotide molecules are used as gene delivery vehicles, as described in WO 90/11092 and U.S. Patent 5,580,859.

- 5 Such gene delivery vehicles can be either *Tim-1* DNA or RNA and, in certain embodiments, are linked to killed adenovirus. Curiel *et al.*, *Hum. Gene. Ther.* 3:147-154, 1992. Other suitable vehicles include DNA-ligand (Wu *et al.*, *J. Biol. Chem.* 264:16985-16987, 1989), lipid-DNA combinations (Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989), liposomes (Wang *et al.*, *Proc. Natl. Acad. Sci.* 84:7851-7855, 10 1987) and microprojectiles (Williams *et al.*, *Proc. Natl. Acad. Sci.* 88:2726-2730, 1991).

- One can increase the efficiency of naked *Tim-1* subgenomic polynucleotide uptake into cells by coating the polynucleotides onto biodegradable latex beads. This approach takes advantage of the observation that latex beads, when incubated with cells in culture, are efficiently transported and concentrated in the perinuclear region of the cells. The 15 beads will then be transported into cells when injected into muscle. *Tim-1* subgenomic polynucleotide-coated latex beads will be efficiently transported into cells after endocytosis is initiated by the latex beads and thus increase gene transfer and expression efficiency. This method can be improved further by treating the beads to increase their hydrophobicity, thereby facilitating the disruption of the endosome and release of *Tim-1* 20 subgenomic polynucleotides into the cytoplasm.

- The invention provides a means of screening test compounds for the ability to which increase or decrease an inflammatory response or to regulate immune responses against tumors, angiogenesis, and chemotaxis of cells such as leukocytes, neutrophils, lymphocytes, hemopoietic progenitors, monocytes, natural killer cells, eosinophils, and 25 dendritic cells. For example, the inflammatory response involves changes in blood vessel caliber and blood flow, increased vascular permeability and the formation of a fluid exudate, and formation of a cellular exudate by emigration of neutrophil polymorphs into the extravascular space. These alterations can be observed morphologically and biochemically.

A cell which expresses the *Tim-1* gene, such as a brain, prostate, or thymus cell, peripheral blood lymphocyte, or a cell which has been genetically engineered to express a *Tim-1* subgenomic polynucleotide, can be contacted with a test compound. The test compound can be a pharmacologic agent already known in the art or may be a compound previously unknown to have any pharmacological activity. The test compound can be naturally occurring or designed in the laboratory. It can be isolated from microorganisms, animals, or plants, and can be produced recombinantly, or synthesized by chemical methods known in the art.

A test compound which decreases *Tim-1* gene expression in the cell is identified as a potential agent for decreasing an inflammatory response, for regulating immune responses against tumors, for increasing angiogenesis or for blocking chemotaxis of cells such as leukocytes, neutrophils, lymphocytes, hemopoietic progenitors, monocytes, natural killer cells, eosinophils, and dendritic cells. A test compound which increases *Tim-1* gene expression in the cell is identified as a potential agent for increasing an inflammatory response, for inhibiting angiogenesis or for increasing chemotaxis of cells such as leukocytes, neutrophils, lymphocytes, hemopoietic progenitors, monocytes, natural killer cells, eosinophils, and dendritic cells. Expression of a *Tim-1* gene can be detected, *inter alia*, by detecting Tim-1 protein immunochemically, using antibodies of the invention, or by detecting *Tim-1* mRNA, as is known in the art. Similar assays can be conducted *in vitro* to detect compounds which specifically decrease *Tim-1* transcription. Comparisons can be made between *Tim-1* gene expression in a cell which has been contacted with the test compound and *Tim-1* gene expression in a cell which has not been contacted with the test compound.

Expression of an endogenous *Tim-1* gene in a cell can be altered by introducing in frame with the endogenous *Tim-1* gene a DNA construct comprising a *Tim-1* targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site by homologous recombination, such that a homologously recombinant cell comprising the DNA construct is formed. The new transcription unit can be used to turn the *Tim-1* gene

on or off as desired. This method of affecting endogenous gene expression is taught in U.S. Patent No. 5,641,670, which is incorporated herein by reference in its entirety.

The targeting sequence is a segment of at least 10, 12, 15, 20, or 50 contiguous nucleotides selected from the nucleotide sequence shown in SEQ ID NO:1. The transcription unit is located upstream of a coding sequence of the endogenous *Tim-1* gene. The exogenous regulatory sequence directs transcription of the coding sequence of the *Tim-1* gene.

The invention provides a therapeutic composition for providing *Tim-1* function to a cell. Wild-type *Tim-1* functions are disclosed above and include involvement in inflammatory responses, regulation of immune responses against tumors, angiogenesis, natural killer cell activation, and chemotaxis. Restoration of wild-type *Tim-1* function can be used to treat tumors, for example by inhibiting angiogenesis. The cell can be any cell of a human which would normally express the wild-type *Tim-1* gene, such as a brain, prostate, thymus, placenta, liver, or kidney cell, or a peripheral blood lymphocyte.

A *Tim-1* therapeutic composition comprises all or a portion of a polynucleotide which encodes all or a portion of a wild-type human *Tim-1* expression product or all or a portion of a *Tim-1* protein and a pharmaceutically acceptable carrier. The *Tim-1* expression product can be, *e.g.*, mRNA or protein. The protein can have the amino acid sequence shown in SEQ ID NO:2. The polynucleotide can have the nucleotide sequence shown in SEQ ID NO:1. Either a full-length *Tim-1* protein or a portion of a *Tim-1* protein can be supplied, if the portion is capable of providing *Tim-1* function to the cell. Portions of *Tim-1* can be assayed for the ability to provide *Tim-1* functions as described above.

Preferably, the therapeutic composition contains an expression construct comprising a promoter and a polynucleotide segment encoding at least six contiguous amino acids of *Tim-1* protein. Within the expression construct, the polynucleotide segment is located downstream from the promoter, and transcription of the polynucleotide segment initiates at the promoter. A more complete description of gene transfer vectors, especially retroviral vectors, is contained in U.S. Serial No. 08/869,309, which is expressly incorporated herein.



Pharmaceutically acceptable carriers are well known to those in the art. Such carriers include, but are not limited to, large, slowly metabolized macromolecules, such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutically acceptable salts can also be used in the composition, for example, mineral salts such as hydrochlorides, hydrobromides, phosphates, or sulfates, as well as the salts of organic acids such as acetates, propionates, malonates, or benzoates. The composition can also contain liquids, such as water, saline, glycerol, and ethanol, as well as substances such as wetting agents, emulsifying agents, or pH buffering agents. Liposomes, such as those described in U.S. 5,422,120, WO 95/13796, WO 91/14445, or EP 524,968 B1, can also be used as a carrier for a therapeutic Tim-1 chemokine composition.

Therapeutic compositions for treating or preventing inflammation and for regulating immune responses against tumors, inducing angiogenesis, and inhibiting cellular chemotaxis are also provided by the present invention. Such therapeutic compositions comprise a pharmaceutically acceptable carrier, as disclosed above, and a reagent which specifically binds to a human *Tim-1* gene or its expression product. The reagent can be, for example, an antibody, an antisense oligomer, or a ribozyme. Reagents which specifically bind to a receptor for a Tim-1 chemokine protein can also be used in the therapeutic composition. In one embodiment of the invention, expression of *Tim-1* is decreased using a ribozyme, an RNA molecule with catalytic activity. See, e.g., Cech, 1987, *Science* 236: 1532-1539; Cech, 1990, *Ann. Rev. Biochem.* 59:543-568; Cech, 1992, *Curr. Opin. Struct. Biol.* 2: 605-609; Couture and Stinchcomb, 1996, *Trends Genet.* 12: 510-515. Ribozymes can be used to inhibit gene function by cleaving an RNA sequence, as is known in the art (e.g., Haseloff *et al.*, U.S. 5,641,673).

The coding sequence shown in SEQ ID NO:1 can be used to generate a ribozyme which will specifically bind to *Tim-1* mRNA. Methods of designing and constructing ribozymes which can cleave other RNA molecules in trans in a highly sequence specific manner have been developed and described in the art (see Haseloff *et al.*, *Nature* 334:585-591, 1988). For example, the cleavage activity of ribozymes can be targeted to

specific RNAs by engineering a discrete "hybridization" region into the ribozyme. The hybridization region contains a sequence complementary to the target RNA and thus specifically hybridizes with the target (see, for example, Gerlach *et al.*, EP 321,201). Longer complementary sequences can be used to increase the affinity of the hybridization sequence for the target. The hybridizing and cleavage regions of the ribozyme can be integrally related; thus, upon hybridizing to the target RNA through the complementary regions, the catalytic region of the ribozyme can cleave the target.

*Tim-1* ribozymes can be introduced into cells as part of a DNA construct, as is known in the art. The DNA construct can also include transcriptional regulatory elements, such as a promoter element, an enhancer or UAS element, and a transcriptional terminator signal, for controlling transcription of the ribozyme in the cells.

Mechanical methods, such as microinjection, liposome-mediated transfection, electroporation, or calcium phosphate precipitation, can be used to introduce the *Tim-1* ribozyme-containing DNA construct into cells in order to decrease *Tim-1* expression. Alternatively, if it is desired that the cells stably retain the DNA construct, it can be supplied on a plasmid and maintained as a separate element or integrated into the genome of the cells, as is known in the art.

Expression of *Tim-1* can also be altered using an antisense oligonucleotide. The sequence of the antisense oligonucleotide is complementary to at least a portion of the coding sequence shown in SEQ ID NO:1. Preferably, the antisense oligonucleotide is at least six nucleotides in length, but can be at least 8, 11, 12, 15, 20, 25, 30, 35, 40, 45, or 50 nucleotides long. Longer sequences, such as the complement of the nucleotide sequence shown in SEQ ID NO:1, can also be used. Antisense oligonucleotides can be provided in an *Tim-1* construct of the invention and introduced into tumor cells, using transfection techniques known in the art.

*Tim-1* antisense oligonucleotides can be composed of deoxyribonucleotides, ribonucleotides, or a combination of both. Oligonucleotides can be synthesized manually or by an automated synthesizer, by covalently linking the 5' end of one nucleotide with the 3' end of another nucleotide with non-phosphodiester internucleotide linkages such

alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamdate, carboxymethyl esters, carbonates, and phosphate triesters. See Brown, 1994, *Meth. Mol. Biol.* 20:1-8; Sonveaux, 1994, *Meth. Mol. Biol.* 26:1-72; Uhlmann *et al.*, 1990, *Chem.*

5 *Rev.* 90:543-583.

Although precise complementarity is not required for successful duplex formation between an *Tim-1* antisense oligonucleotide and the complementary coding sequence of *Tim-1*, antisense oligonucleotides with no more than one mismatch are preferred. One skilled in the art can easily use the calculated melting point of an *Tim-1* antisense-sense pair to determine the degree of mismatching which will be tolerated between a particular antisense oligonucleotide and a particular coding sequence of *Tim-1*.

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*Tim-1* antisense oligonucleotides can be modified without affecting their ability to hybridize to an *Tim-1* coding sequence. These modifications can be internal or at one or both ends of the antisense oligonucleotide. For example, internucleoside phosphate linkages can be modified by adding cholesteryl or diamine moieties with varying numbers of carbon residues between the amino groups and terminal ribose. Modified bases and/or sugars, such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide in which the 3' hydroxyl group or the 5' phosphate group are substituted, can also be employed in a modified antisense oligonucleotide. These modified oligonucleotides can be prepared by methods well known in the art. Agrawal *et al.*, *Trends Biotechnol.* 10:152-158, 1992; Uhlmann *et al.*, *Chem. Rev.* 90:543-584, 1990; Uhlmann *et al.*, *Tetrahedron. Lett.* 215:3539-3542, 1987.

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Antibodies of the invention which specifically binds to the *Tim-1* chemokine protein can also be used to alter *Tim-1* expression. Methods of preparing such antibodies are disclosed above. In addition, reagents which specifically bind to a receptor for *Tim-1* chemokine protein, such as antibodies, can be used in a therapeutic composition to prevent the interaction of *Tim-1* chemokine protein with its receptor. Antibodies which specifically bind to *Tim-1* receptors can be prepared, as described above. Other chemical

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reagents can be identified using candidate reagents in competitive binding assays with labeled Tim-1 chemokine proteins or polypeptides of the invention, as is known in the art.

Preferably, the reagent used to decrease expression of the *Tim-1* gene, whether ribozyme, antisense nucleotide sequence, or antibody, decreases expression of the *Tim-1* gene by at least 50%, 60%, 70%, or 80%. Most preferably, expression of the *Tim-1* gene is decreased by at least 90%, 95%, 99%, or 100%. The amount of a particular *Tim-1* composition which is effective to decrease expression of *Tim-1* can be assessed using methods well known in the art, such as hybridization of nucleotide probes to *Tim-1* mRNA or detection of a Tim-1 chemokine protein using antibodies of the invention.

Therapeutic compositions of the invention are prepared as injectables, either as liquid solutions or suspensions; however, solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The compositions can also be formulated into enteric coated tablets or gel capsules according to known methods in the art, such as those described in U.S. 4,853,230, EP 225,189, AU 9,224,296, and AU 9,230,801.

Inflammatory responses to a variety of stimuli, such as heart attacks or strokes, infection, physical trauma, UV or ionizing radiation, burns, frostbite, or corrosive chemicals, can be treated by administration of a therapeutic *Tim-1* composition. Inflammation associated with diseases such as lupus, rheumatoid arthritis, Crohn's disease, ulcerative colitis, or hepatitis, can also be treated. Administration of the *Tim-1* therapeutic agents of the invention can include local or systemic administration, including injection, oral administration, particle gun, or catheterized administration, and topical administration. Various methods can be used to administer a therapeutic *Tim-1* composition directly to a specific site in the body. For example, tumors or areas of inflammation can be located and a therapeutic *Tim-1* composition injected several times in these locations. Alternatively, arteries which serve a tumor or a region which is inflamed can be identified, and the therapeutic composition injected into the artery. A therapeutic *Tim-1* composition can be directly administered to the surface of an inflamed region or a tumor, for example, by topical application of the composition. Combination therapeutic

agents, including more than one reagent capable of inhibiting *Tim-1* expression, can be administered simultaneously or sequentially.

Receptor-mediated targeted delivery of therapeutic compositions containing *Tim-1* subgenomic polynucleotides to specific tissues can also be used. Receptor-mediated DNA  
5 delivery techniques are described in, for example, Findeis et al. (1993), *Trends in Biotechnol.* 11, 202-05; Chiou et al. (1994), GENE THERAPEUTICS: METHODS AND APPLICATIONS OF DIRECT GENE TRANSFER (J.A. Wolff, ed.); Wu & Wu (1988), *J. Biol. Chem.* 263, 621-24; Wu et al. (1994), *J. Biol. Chem.* 269, 542-46; Zenke et al. (1990), *Proc. Natl. Acad. Sci. U.S.A.* 87, 3655-59; Wu et al. (1991), *J. Biol. Chem.* 266, 338-42.

10 Alternatively, a *Tim-1* therapeutic composition can be introduced into human cells *ex vivo*, and the cells then replaced into the human. The removed cells can then be contacted with the *Tim-1* therapeutic composition utilizing any of the above-described techniques, followed by the return of the cells to the human, preferably to or within a tumor or the vicinity of the inflammation.

15 Both the dose of the *Tim-1* composition and the means of administration can be determined based on the specific qualities of the therapeutic composition, the condition, age, and weight of the patient, the progression of the disease, and other relevant factors. If the composition contains *Tim-1* chemokine protein or polypeptide, effective dosages of the composition are in the range of about 5 µg to about 50 µg/kg of patient body weight,  
20 about 50 µg to about 5 mg/kg, about 100 µg to about 500 µg/kg of patient body weight, and about 200 to about 250 µg/kg.

Effective amounts of therapeutic compositions containing *Tim-1* subgenomic polynucleotides, ribozymes, or antisense oligonucleotides range from about 100 ng to about 200 mg of DNA for local administration. Concentration ranges of about 500 ng to  
25 about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA can also be used. Factors such as method of action and efficacy of transformation and expression are considerations that will effect the dosage required for ultimate efficacy of the *Tim-1* subgenomic polynucleotides, ribozymes, or antisense oligonucleotides. Where greater expression is desired over a larger area of tissue, larger

amounts of the therapeutic composition or the same amounts readministered in a successive protocol of administrations, or several administrations to different areas adjacent or close to a tumor or an area of inflammation, may be required to effect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will  
5 determine specific ranges for optimal therapeutic effect.

The invention provides a knock-out mammal whose endogenous *Tim-1* gene is not expressed. Methods of making knock-out mammals are well known in the art. The mammal can be any experimental mammal, such as a mouse, rat, or rabbit; however, a mouse is preferred. The endogenous wild-type *Tim-1* gene of the mammal can be deleted  
10 entirely, resulting in an absence of Tim-1 chemokine protein in the mammal. Alternatively, mutations such as deletions, insertions, missense substitutions, or inversions, can be introduced into the *Tim-1* gene. Such mutations result in expression of truncated or otherwise aberrant forms of Tim-1 chemokine protein in the knock-out mammal.

15 Knock-out mammals of the invention are useful as model systems for studying the effects of drugs in the absence of wild-type Tim-1 chemokine protein or in the presence of altered forms of the Tim-1 chemokine protein in the mammal. Knock-out mammals can also be used to develop therapeutic treatments for diseases associated with alterations in *Tim-1* gene expression.

20 A *Tim-1* subgenomic polynucleotide can also be delivered to subjects for the purpose of screening test compounds for those which are useful for enhancing transfer of *Tim-1* subgenomic polynucleotides to the cell or for enhancing subsequent biological effects of *Tim-1* subgenomic polynucleotides within the cell. Such biological effects include hybridization to complementary *Tim-1* mRNA and inhibition of its translation,  
25 expression of a *Tim-1* subgenomic polynucleotide to form *Tim-1* mRNA and/or Tim-1 protein, and replication and integration of a *Tim-1* subgenomic polynucleotide. The subject can be a cell culture or an animal, preferably a mammal, more preferably a human.

Test compounds which can be screened include any compounds, whether natural products or synthetic, which can be administered to the subject. Libraries or mixtures of

compounds can be tested. The compounds can be those for which a pharmaceutical effect is previously known or unknown. The compounds can be delivered before, after, or concomitantly with a *Tim-1* subgenomic polynucleotide. They can be administered separately or in admixture with a *Tim-1* subgenomic polynucleotide.

5           Integration of a delivered *Tim-1* subgenomic polynucleotide can be monitored by any means known in the art. For example, Southern blotting of the delivered *Tim-1* subgenomic polynucleotide can be performed. A change in the size of the fragments of a delivered polynucleotide indicates integration. Replication of a delivered polynucleotide can be monitored *inter alia* by detecting incorporation of labeled nucleotides combined  
10           with hybridization to a *Tim-1* probe. Expression of a *Tim-1* subgenomic polynucleotide can be monitored by detecting production of *Tim-1* mRNA which hybridizes to the delivered polynucleotide or by detecting *Tim-1* protein. *Tim-1* protein can be detected immunologically. Thus, the delivery of *Tim-1* subgenomic polynucleotides according to the present invention provides an excellent system for screening test compounds for their  
15           ability to enhance transfer of *Tim-1* subgenomic polynucleotides to a cell, by enhancing delivery, integration, hybridization, expression, replication or integration in a cell *in vitro* or in an animal, preferably a mammal, more preferably a human.

          The following material was deposited with the American Type Culture Collection 10801 University Blvd., Manassas, VA 20110-2209, U.S.A., under the accession number  
20           indicated.

<u>Name</u>	<u>Deposit Date</u>	<u>Accession No.</u>	<u>CMCC Accession No.</u>
pCR.HCSC1.HIS	May 27, 1998	209935	4818

25           This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The deposit will be maintained for a period of 30 years following issuance of this patent, or for the enforceable life of the patent, whichever is greater. Upon issuance of the patent, the deposit will be available to the public from the ATCC without restriction.

The deposit is provided merely as a convenience to those of skill in the art, and is not an admission that a deposit is required under 35 U.S.C. § 112. The sequence of the polynucleotides contained within the deposited material, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and  
5 are controlling in the event of any conflict with the written description of sequences herein. A license may be required to make, use, or sell the deposited material, and no such license is granted hereby.

The following is provided for exemplification purposes only and are not intended to limit the scope of the invention which has been described in broad terms above. The  
10 complete contents of all references cited in this disclosure are expressly incorporated herein.

#### EXAMPLE 1

This example demonstrates distribution of *Tim-1* mRNA in the stomach wall and  
15 small intestine.

*In situ* hybridization was performed essentially as described in Gunn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 95, 258-63, 1998. Briefly, 0.5  $\mu$ m paraffin sections of the mouse stomach wall and small intestine were deparaffinized, fixed in 4% paraformaldehyde, and treated with proteinase K. After washing in 0.075 M sodium chloride, 0.0075 M sodium  
20 citrate, pH 7, sections were covered with hybridization solution, prehybridized for 1-3 hours at 55 °C, and then hybridized overnight with either sense or antisense <sup>35</sup>S-labeled riboprobe transcribed from the murine analog of *Tim-1*. Following the hybridization step, the sections were washed at high stringency, dehydrated, and coated with NTB2 photographic emulsion (Kodak). Following a 2-8  
25 week exposure at 4 °C, the sections were developed and counterstained with hematoxylin and eosin.

Cells within the crypts of the stomach wall and the tips of the villi of the small intestine were labeled with the *Tim-1 in situ* probes, indicating that *Tim-1* mRNA is expressed in these locations.



**CLAIMS**

1. An isolated and purified Tim-1 chemokine protein having an amino acid sequence which is at least 85% identical to an amino acid sequence as shown in SEQ ID NO:2, wherein percent identity is determined using a Smith-Waterman algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

2. The isolated and purified Tim-1 chemokine protein of claim 1 which has the amino acid sequence shown in SEQ ID NO:2.

3. An isolated and purified Tim-1 chemokine polypeptide comprising at least 8 contiguous amino acids selected from an amino acid sequence as shown in SEQ ID NO:2.

4. A fusion protein comprising a first protein segment and a second protein segment fused together by means of a peptide bond, wherein the first protein segment comprises at least 8 contiguous amino acids of a Tim-1 chemokine protein selected from an amino acid sequence as shown in SEQ ID NO:2.

5. A preparation of antibodies which specifically bind to a Tim-1 chemokine protein having an amino acid sequence as shown in SEQ ID NO:2.

6. a cDNA molecule which encodes a Tim-1 protein having an amino acid sequence which is at least 85% identical to SEQ ID NO:2, wherein percent identity is determined using a Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

7. A cDNA molecule which encodes at least 8 contiguous amino acids of SEQ ID NO:2.

8. The cDNA molecule of claim 7 which encodes SEQ ID NO:2.

9. The cDNA molecule of claim 8 which comprises SEQ ID NO:1.

10. A cDNA molecule comprising at least 12 contiguous nucleotides of SEQ ID NO:1.

11. A cDNA molecule which is at least 85% identical to the nucleotide sequence shown in SEQ ID NO:1, wherein percent identity is determined using a Smith-

Waterman homology search algorithm as implemented in a MPSRCH program using an affine gap search with a gap open penalty of 12 and a gap extension penalty of 1.

12. An isolated and purified subgenomic polynucleotide comprising a nucleotide sequence which hybridizes to SEQ ID NO:1 after washing with 0.2X SSC at 65 °C, wherein the nucleotide sequence encodes a Tim-1 protein having the amino acid sequence of SEQ ID NO:2.

13. The isolated and purified subgenomic polynucleotide of claim 12 which is contained in an ATCC deposit with Accession No. 209935.

14. A construct comprising:  
a promoter; and  
a polynucleotide segment encoding at least 8 contiguous amino acids of a Tim-1 protein as shown in SEQ ID NO:2, wherein the polynucleotide segment is located downstream from the promoter, wherein transcription of the polynucleotide segment initiates at the promoter.

15. A host cell comprising a construct which comprises:  
a promoter and:  
a polynucleotide segment encoding at least 8 contiguous amino acids of a human Tim-1 protein having an amino acid sequence as shown in SEQ ID NO:2.

16. A recombinant host cell comprising a new transcription initiation unit, wherein the new transcription initiation unit comprises in 5' to 3' order:

- (a) an exogenous regulatory sequence;
- (b) an exogenous exon; and
- (c) a splice donor site,

wherein the new transcription initiation unit is located upstream of a coding sequence of a *Tim-1* gene as shown in SEQ ID NO:1, wherein the exogenous regulatory sequence controls transcription of the coding sequence of the *Tim-1* gene.

17. A polynucleotide probe comprising at least 12 contiguous nucleotides of SEQ ID NO:1.

18. The polynucleotide probe of claim 16 which comprises a detectable label.

**SEQUENCE LISTING**

SEQ ID NO:1 (*Tim-1* coding sequence)

ATGTCCCTGCTCCACGCCGCGCCCCTCCGGTCAGCATGAGGCTCCTGGCGGC  
CGCGCTGCTCCTGCTGCTGCTGGCGCT  
GTACACCGCGCGTGTGGACGGGTCCAAATGCAAGTGCTCCCGGAAGGGACCC  
AAGATCCGCTACAGCGACGTGAAGAAGC  
TGGAATGAAGCCAAAGTACCCGCACTGCGAGGAGAAGATGGTTATCATCAC  
CACCAAGAGCGTGTCCAGGTACCGAGGT  
CAGGAGCACTGCCTGCACCCCAAGCTGCAGAGCACCAAGCGCTTCATCAAGT  
GGTACAACGCCTGGAACGAGAAGCGCAG  
GGTCTACGAAGAATAG

SEQ ID NO:2 (*Tim-1* protein)

MSLLPRRAPPVSMRLAALLLLLLALYTARVDGSKCKCSRKGPKIRYSDVKKLE  
MKPKYPHCEEKMVIITTKSVSRYG  
QEHCLHPKLQSTKRFIKWYNAWNEKRRVYEE\*

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 98/26546

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC 6 C12N15/19 C07K14/52 C12Q1/68 C07K16/24

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C12N C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35010 A (HUMAN GENOME SCIENCES INC (US); NI J.; GENTZ R.L.; SU J.Y.; LI H.) 25 September 1997 see abstract see page 1 - page 6 see page 65 - page 68 see page 71 - page 74; claims see figures 1,2	1-18
P, X	WO 98 32858 A (SCHERING CORP (US); MATTSON; SOTO-TREJO; HEDRICK; GORMAN; ZLOTNIK) 30 July 1998 see abstract see page 62, line 4-13 see page 76 - page 77 see page 101 - page 103; claims	1-18
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
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Date of the actual completion of the international search

7 April 1999

Date of mailing of the international search report

20/04/1999

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PCT/US 98/26546

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>Database EMBL, ID HS1301003  Accession number AA505654  4 July 1997  99% identity with Seq.ID:1 nt.51-336  XP002099074  see the whole document</p> <p style="text-align: center;">---</p>	6-15
A	<p>EP 0 807 439 A (SMITHKLINE BEECHAM CORP  (US); WHITE J.R.; PELUS L.M.)  19 November 1997  see page 3; table 1</p> <p style="text-align: center;">---</p>	
A	<p>BAGGIOLINI M. ET AL.: "Human chemokines:  an update"  ANNUAL REVIEW OF IMMUNOLOGY,  vol. 15, 1997, pages 675-705, XP002055737</p> <p style="text-align: center;">---</p>	
A	<p>MILLER M.D. ET AL.: "Biology and  biochemistry of the chemokines: a family  of chemotactic and inflammatory cytokines"  CRITICAL REVIEWS IN IMMUNOLOGY,  vol. 12, no. 1,2, 1992, pages 17-46,  XP002049224</p> <p style="text-align: center;">-----</p>	

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 98/26546

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W0 9832858 A	30-07-1998	AU 6132698 A	18-08-1998
EP 0807439 A	19-11-1997	JP 10053533 A	24-02-1998